IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Steffen GOLETZ et al. Examiner: GUSSOW, Anne

Serial No.: 10/540,479 Group Art Unit: 1643

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Title: **RECOGNITION MOLECULES FOR THE TREATMENT AND DETECTION OF TUMOURS**

DECLARATION UNDER 37 C.F.R. § 1.132

Commissioner for Patents
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Sir:

I, A. Daniel Gyle being duly warned, declare that

1. I am a coinventor named in the above-identified application. I am an employee of the assignee of the application, Nemod Biotherapeutics GmbH & Co. KG, of Berlin, GERMANY and thus would receive some compensation related to sales if the invention is ever commercialized

2. The following experiments were conducted by me or under my supervision:

.....

Example 15

Goal: Tumor therapy for the reduction of MUC1-positive tumors in *in vivo* tumor models using non-labeled recognition molecules which specifically recognize glycosylated MUC1 tumor epitope.

Methods: The therapeutic and a prevention potential of the non-labeled cIgG-Panko2 was investigated in a (a) ZR-75-1 tumor model and a (b) SK-LC-4 (human lung cancer cell line) model.

(a) Therapeutic model:

As the ZR-75-1 tumor model 107 MUC-1 positive ZR-75-1, human breast cancer cells (see also Examples 9 and 12 of the present application) were injected subcutaneously into the flank of female SCID mice of 6-9 weeks age. The tumor was allowed to grow to about 100 mm^3 prior to antibody administration. Each tumor-bearing mouse (skin xenografted SCID mouse) received two doses of cIgG-Panko2 per week intravenously into the tail vein over a 4 week period (administration at day 9, 12, 15, 19, 22, 26, 29, and 33

post tumor cell injection; 8 doses in total). Two different doses of cIgG-Panko2 in PBS (1 mg/kg and 10 mg/ kg) were used in two different groups and PBS was used in the control group, with 8 mice per group (n=8). Tumor size was determined over 54 days as indicated in graph and the analysis was performed:

(i) Calculation of the tumor volumes:
tumor volume (min) = 0.5 x (major diameter) x (minor diameter^2)

(ii) Statistical analysis bytwo-tailed unpaired test.

Results are shown in Fig. 17 (A)

Conclusions: The example shows the strong anti-tumor potential of the non-labeled (naked) antibody of the invention *in vivo*, even at a low concentration and even in the skin xeno-transplant model, since the model is based on utilization of human cells and SCID mice with a limited immune effector system.

(b) Prevention model

As the SK-LC-4 tumor model 10^7 MUC1-positive ZR-75-1, human lung cancer cells were injected subcutaneously into the flank of female SCID mice of 6-9 weeks age, 3 hours prior to the first antibody administration. Groups of mice (mice/ group) were treated either with cIgG-Panko2 (1 or 10 mg/kg) or control PBS. After 12 days weeks the tumor was grown to about 100 mm^3. The mice were injected with two doses of cIgG-Panko2 per week for 4 weeks (8 doses in total); dose of 1 mg/ kg and 10 mg/kg, respectively. PBS was used in the control group. Each group consisted of 8 mice (n=8). Administration was intravenously performed into the tail vein. Tumor size was determined over 54 days as indicated in graph and the analysis was performed:

(i) Calculation of the tumor volumes:
tumor volume (mm) = 0.5 x (major diameter) x (minor diameter^2)

(ii) Statistical analysis by twotailed unpaired test.

Results are shown in Fig. 17 (B)

Conclusions: The example shows the strong anti-tumor potential of the non-labeled (naked) antibody of the invention *in vivo* for the therapeutic and the prevention setting, even in the skin xeno-transplant model. The model is known for unfavorable accessibility resulting from the use of human cells and SCID mice with a limited immune effector system.

This proves the suitability of the antibodies of the inventionin therapeutic or prophylactic applications

Fig. 17A:

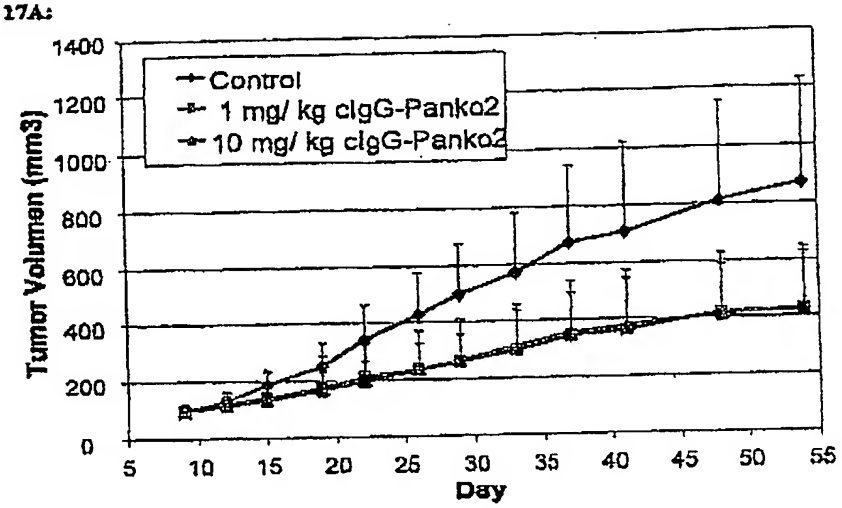


Fig. 17B:

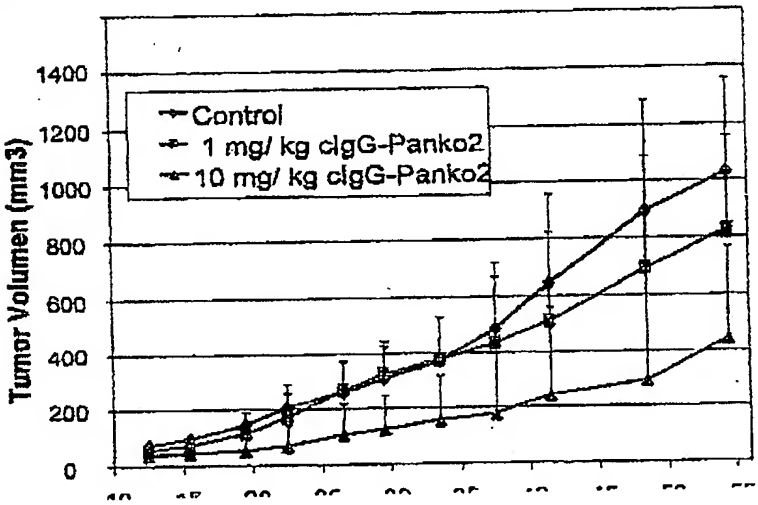


Fig. 17: Inhibition of tumor growth in tumor-bearing mice after treatment with the non-labeled recognition molecule cIgG-Panko2.

Fig 17.A: 9 days after subcutaneous injection of 10^7 human ZR-75-1 cancer cells into SCID mice and tumor growth of about 100 mm^3, groups of 8 mice (n=8) were treated with 1 mg/kg clgG-Panko2 or 10 mg/kg clgGPanko2, or PBS as control, respectively. 8 doses were administered twice a week over 4 week intravenously into the tail vain and tumor growth was monitored as indicated over 54 days.

Fig 17.B: 3 hours prior to antibody administration 10^7 human SIC-LC-4 cancer cells were injected subcutaneously into SCID mice. Groups of 8 mice (n=8) were treated with 1 mg/kg clgG-Panko2 or 10 mg/kg clgGPanko2, or PBS as control, respectively. 8 doses were administered twice a week over 4 week intravenously into the tail vain and tumor growth was monitored as indicated over 54 days.

Example 16

Goal: Investigation of antibody-dependent cell cytotoxicity of cIgG1-Panko2 using a europium assay using primary human macrophage activated killer cells as effector cells

Methods: Human donor derived macrophages were differentiated for seven days with GM-CSF and for the last 18h additionally with IFN-γ and incubated with the europium-labeled target cells and cIgG1Panko2. Briefly, tumor cells were harvested with trypsin/EDTA and washed with PBS. $5x10^6$ tumor cells were spun down, re-suspended in cold 800µl europium buffer, transferred into electroporation cuvettes (Eppendorf, depth 4mm) and incubated on ice for 10min. Electroporation was done in an Multiporator (Eppendorf) with 1 pulse for 30µs at 710V (ZR-751TF, MCF-7) or 1100V (MT-3). The cuvettes were incubated on ice for 10min, cells were transferred into 50ml RPMI/5% FCS/1 % L-glutamine spun down, and washed for another 4 times with 15 ml medium. Tumor cells were re-suspended in MAK cell medium (Invitrogen) and counted. Tumor cells were diluted at 5×10^4 cells per ml in MAK cell medium with 10% FCS or with 50% autologous serum. MAK cells were used at 6.25×10^6 cells/ml for E:T ratios of 100:1 or at 1.9×10^6 cells/ml for E:T ratios of 30:1. Antibody (Ab) dilutions were done in MAK cell medium.

The following volumes were distributed into 96 well roundbottom microtiter plates:

20µl antibody solution

80µl/well MAK cells

100µl/well europium-labeled tumor cells

For determination of spontaneous europium release (SR) 100µl of labeled tumor cells were incubated with 100µl MAK cell medium, for determination of maximal release (MR) with 100µl ethanol. For determination of background release (BR) 100µl of supernatant from europium-labeled tumor cells were incubated with 100µl MAK cell medium. Control samples were performed in 6 parallel wells, experimental samples (ExpR) in triplicates. After 4h or overnight (o.n.) (18-20h) incubation, plates were spun down, and 25µl supernatant were transferred into 200µl enhancement solution into 96 well flat bottom micro-titer plates. Europium release was determined at a temporally delayed fluorometer (Victor2, Perkin Elmer).

Spontaneous release was calculated according to the following formula:

% spontaneous release =100 x (SR [counts]- BR [counts])/ (MR [counts]– BR [counts])

For an assay to be evaluated, spontaneous release had to be <30%.

Specific release was calculated according to the following formula:

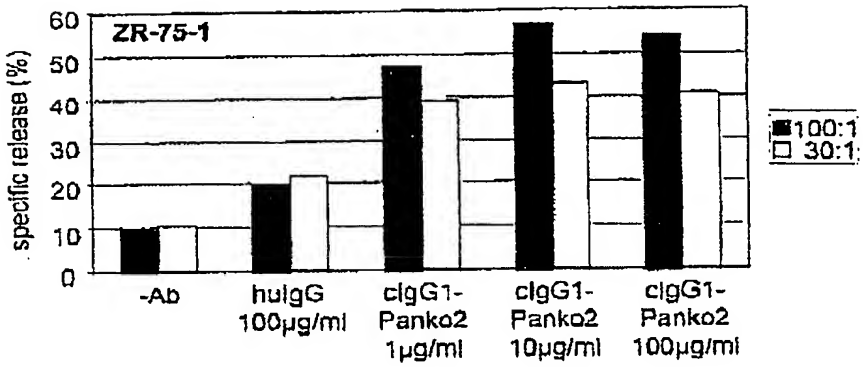
% specific release = 100 x (ExpR [counts]- SR [counts])/ (MR [counts]- SR [counts]).

Europium release assays were performed at an E:T ratio of 100:1, in some cases additionally at an E:T ratio of 30:1. Incubations for 4h or o.n. (16-20h) were performed at 37°C, 5% CO_2.

Observation: Chimeric IgG1-Panko2 triggers strong ADCC activity of human macrophages in a concentration-dependent and antigen-specific manner. A strong lysis was observed with the TA-MUC1 positive cell lines ZR-75-1 and MCF-7 while the MUC1-negative cell line MT-3 is not lysed by the macrophages after incubation with cIgG1-Panko2 (see Fig. 1).

Conclusion: These studies show that the molecules of the invention, for instance, chimeric antibodies cIgG-Panko 1 and cIgG-Panko 2, participate in antigen-dependent cell-mediated cytotoxicity (ADCC). After recognition of the MUC1 tumor epitope by the antibodies, human effector cells were added (human peripheral blood cells). The human effector cells then recognize the chimeric antibodies and kill the tumor cells. This is the usual mechanism of ADCC-dependent immune response and the mechanism for antibody-based tumor treatment. Moreover, these data corroborate with the experimental evidence provided in Fig. 15a of the present specification, wherein chimeric antibodies cIgG-Panko1 and cIgG-Panko 2 are deemed to be highly effective in marking tumor cells for killing by the human peripheral blood cells.

A)



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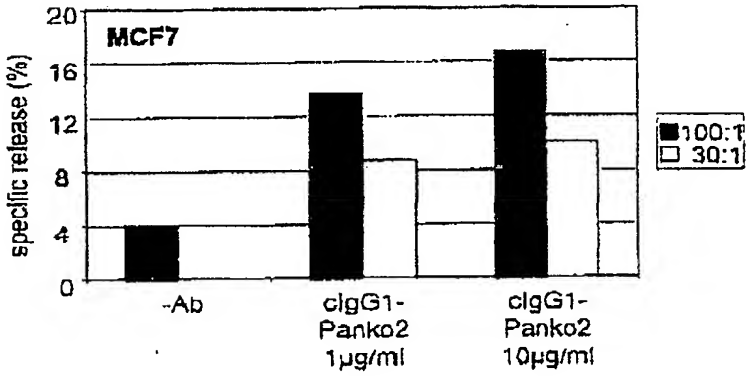


C)

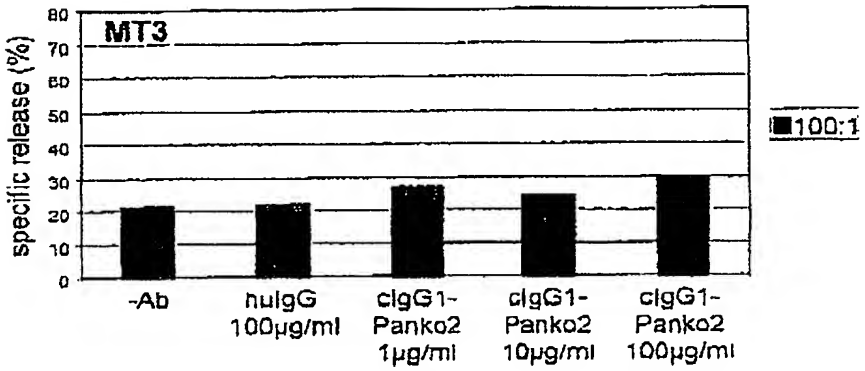


Fig. 1. Europium release assay with primary human macrophage killer cells as effector cells and the MUC1-positive tumor cell lines ZR-75-1 (A), MCF-7 (B) and the MUC1-negative tumor cell line MT-3 (C) as target cells and different concentrations of cIgG1-Panko2.

Example 17

Goal: Anti-tumor activity: Detection of antibody mediated complement-dependent cellular cytotoxicity against tumor cells with recognition molecules, which specifically recognize the glycosylated MUC1 tumor epitope, in an in vitro model.

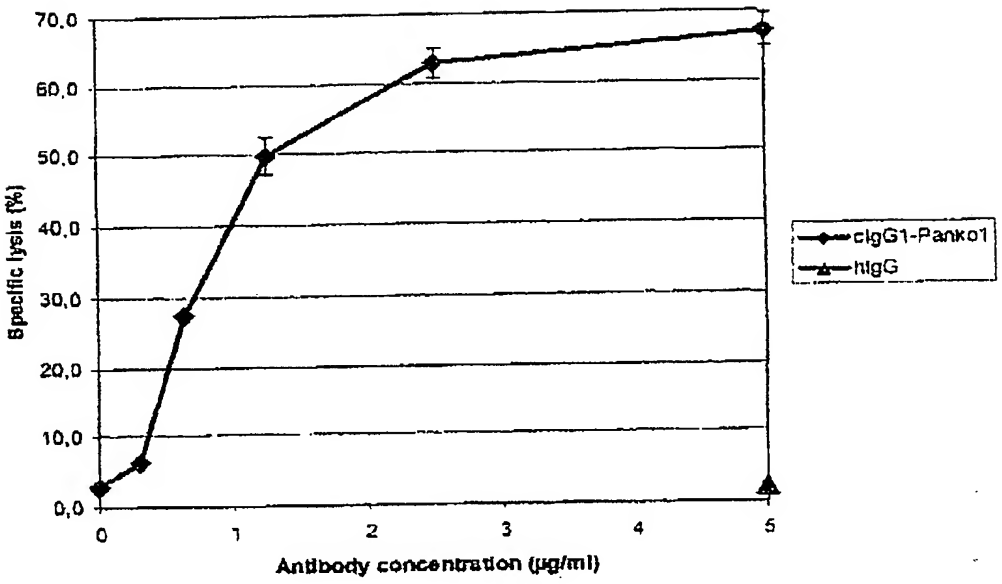
Methods: The complement-dependent cellular cytotoxicity (CDC) of chimeric IgGI-Pankol and IgG1Panko2 was investigated in a europium releaseassay. Eu3+ -loaded target cells (ZR-75-1, 3 x 106) were prepared as described above.

Thereafter, the cells were seeded in a 96-well round-bottom plate (Nunc; 5 x 103 cells in 100µl per well). Following addition of 20µl of antibodies at varying concentration (0.5 to 50 µl/ml final concentration in 200µl incubation volume) or the corresponding controls (medium, isotype control human IgG), cells were incubated half an hour at room temperature. Thereafter, 10µl per well baby rabbit complement (Cedarline, 1:5 to 1:10 diluted) were added. 10 µl RPMI/FCS with no complementas added to determine spontaneous release (SR). Maximum release (MR) was determined after complete lysis of the target cells with ethanol or 1 % TritonX-100. Basal release (BR) was determined by incubation of the supernatant of europium labeled target cells. Following incubation in an incubator at 37°C for 3 to 3.5 hours, the plate as centrifuged at 500 x g for 5 minutes, and 25µl of each sample was pipetted in 200µl per well of enhancement solution (Perkin-Elmer Wallace). Following incubation or 10 min at room temperature, the time resolved fluorescence was determined at thefluorometer Victor2 (Perkin-Elmer Wallace).

Spontaneous release was calculated according to the following formula:
% spontaneous release =100 x (SR [counts] - BR [counts])/(MR [counts] - BR [counts]) For an assay to be evaluated, spontaneous release had to be <30%.
Specific release was calculated according to the following formula:
% specific release = 100 x (ExpR [counts]- SR [counts])/ (MR [counts] - SR [counts]).

Observation: As shown in Fig. 2, chimeric IgG1-Pankol and IgG1-Panko2 specifically trigger potent complement dependent cytotoxicity of human tumor cells. clgG1-Panko2 without complement or hIgG as a negative control do not induce complement-dependent cytotoxicity of ZR-75-1 cells.

A)



B)

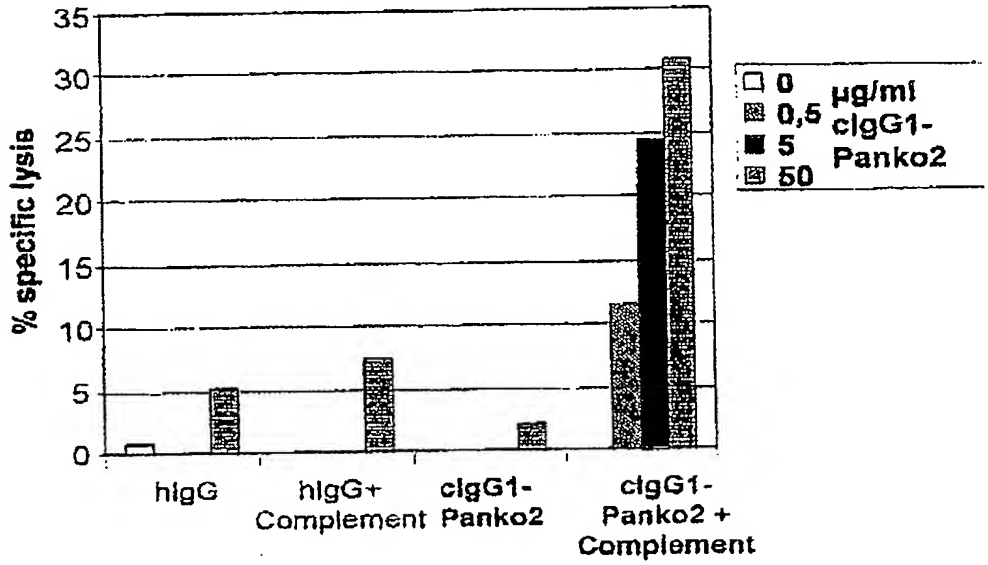


Fig. 2 shows that potent complement-dependent cytotoxicity of ZR-75-1 cells is triggered by chimeric IgG1-Pankol (A) and IgG1-Panko2 (B).

Example 18

Goal: Study of anti-tumor activity in human setting: Detection of antibody mediated phagocytosis of tumor cells with primary human macrophages with recognition molecules, which specifically recognize the glycosylated MUC1 tumor epitope, in an *in vitro* model (conjugate formation assay: CFA).

Methods:

Tumor cell staining with PKH26

$1x10^7$ to $2x10^7$ tumor cells were washed twice in PBS, re-suspended in 1 ml diluent C, 1 ml PKH26 was added at a concentration of 12×10^{-6} M for ZR-751, MCF-7 and MT-3. After 3min incubation at RT, staining was stopped by adding 2ml FCS for 1 min at RT. Medium (4ml, RPMI supplemented with 1% L-glutamine, 10 % FCS) was added, cells were spun down and washed 4 times with medium. Tumor cells were incubated o.n. at 37°C, 5% CO2 to allow the release of excess PKH-26. Optimization of PKH-26 staining was performed with half of the cell numbers and volumes.

CFA:

PKH-26 labeled tumor cells were harvested with trypsin/EDTA, washed once with PBS and re-suspended at 0.8×10^6 cells/ml in MAK cell medium (Invitrogen) in the presence or absence of recombinant antibody molecule compositions. Tumor cells (250µl, 0.2×10^5 cells) were seeded in non-adherent polypropylene tubes. MAK cells were prepared according to Boyer et al. (*Exp. Hematol.*, vol. 27, 751-76, 1999), washed and re-suspended at 1.6×10^6 cells/ml. 250µl MAK cells (0.2×10^5 cells) were added to PKH-26 labeled tumor cells (E:T ratio 2:1) Individual samples were incubated in duplicates at 4°C and 37°C/5% CO2 for 3 h or o.n. (18 -20h).

After incubation at 3h or o n. cells were washed with PBS and stained with CD11c-FITC (1:18.5) + 7-AAD (1:500) in PBS/10% FCS. Cells were washed with PBS, re-suspended in 400µl PBS. Acquisition was done on 10,000 cells in an Epics XL flow cytometer(Beckman Coulter). Percentage of co-localized MAK cells was determined as percentage of PKH-26 positive cells in the cell population gated for all viable CD1 1 c-FITC positive cells. Phagocytosis of chimeric IgG1-Panko2 was analyzed in a conjugate formation assay with macrophage activated killer cells (MAK cells) and the TA-MUC-1 positive tumor cell lines ZR75-1 and MCF-7. Therefore, human donor derived macrophages were differentiated for seven days with GM-CSF and for the last 18h additionally with IFN-γ. MAK cells were co-incubated with PKH26 (red fluorescent dye)-labeled tumor cells in the presence or absence of cIgG1-Panko2 for 3h or o.n.. MAK cells were labeled with a FITC-conjugated anti-CD11c antibody and the samples were analyzed by flow cytometry. Co-localization was measured as percentage of PKH-26 and FITC double positive cells among the cell population (see Fig. 3).

Fig. 3

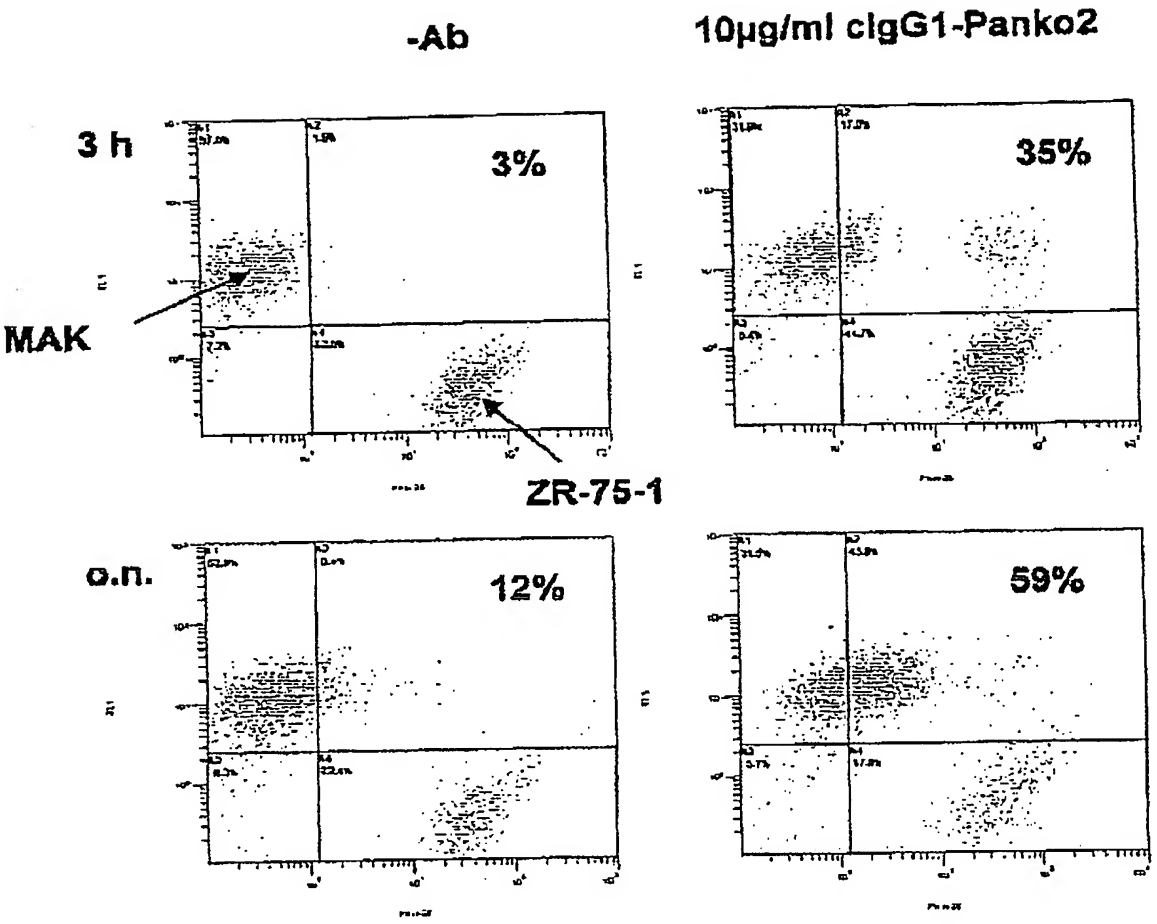


Fig. 3 shows results from a conjugate formation assay with MAK cells and ZR-75-1 in the presence or absence of cIgG1-Panko2.

Observation: It was further observed that cIgG1-Panko2 was capable of mediating MAK co-localization with the TA-MUC1-positive tumor cell lines ZR-75-1 and MCF-7. MAK co-localization with cigG1 Panko2 was antigen-specific as no increase in MAK co-localization could be detected with human IgG as control antibody or with the MUC1 negative tumor cell line MT-3 (for details see Fig. 4).

Fig. 4

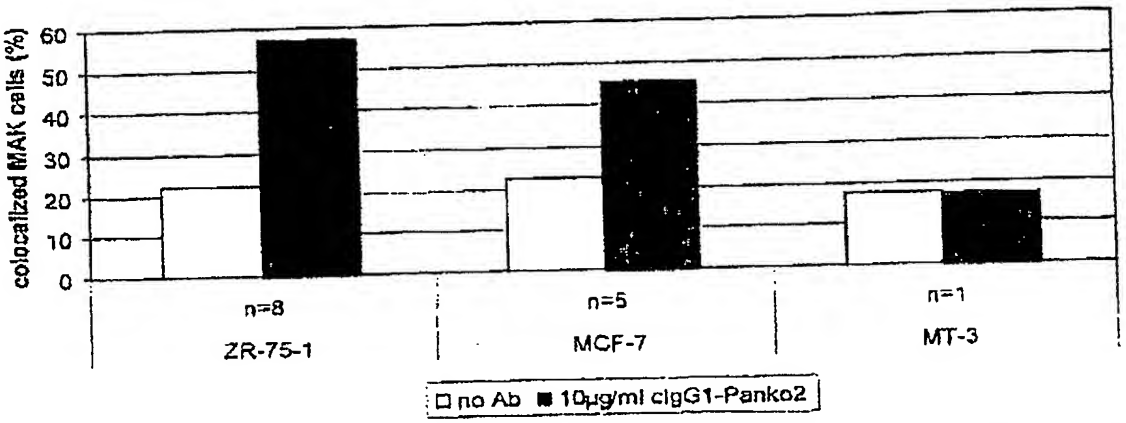


Fig. 4 shows results of a conjugate formation assay with clgG1-Panko2 and MAK cells derived from different healthy human donors after over night incubation (n=number of donors tested).

Co-localization studies

Co-localization was dramatically enhanced after incubation at 37°C compared to 4°C incubations suggesting an active process being responsible for the co-localization rather than passive adhesion of tumor cells and MAK cells in the presence of clgG1-Panko2. By confocal microscopy, it was shown that the conjugates of MAK cells and tumor cells measured in flow cytometry analysis indeed represent macrophages ingesting tumor cells as shown in Fig. 5. Therefore, clgG1-Panko2 induces tumor cell phagocytosis of primary human macrophages.

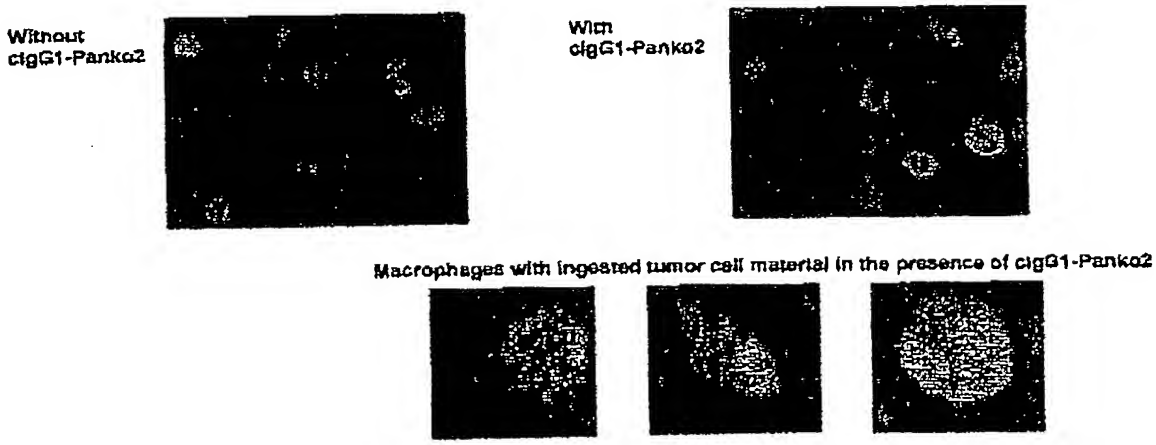


Fig. 5 shows images of MAK cells and ZR-75-1 following incubation with or without clgG 1-Panko2. Images were obtained using confocal microscopy.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

6.6.08

Date